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# Prognostic Significance of DNA Ploidy, S-Phase Fraction, and P-Glycoprotein Expression in Colorectal Cancer

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**Background and Objectives:** Parameters that allow prediction of the disease course in colorectal cancer would aid the development of improved treatment strategies. For this reason, we evaluated the prognostic value of flow cytometric DNA ploidy and S-phase fraction (SPF) and P-glycoprotein (Pgp) expression in this type of tumor.
**Methods:** The prognostic significance of DNA ploidy, SPF, and Pgp expression on paraffin-embedded sections from 107 patients with colorectal carcinoma was determined. The mean follow-up was 36.6 months (range = 3–72 months). DNA ploidy and SPF were evaluated by flow cytometry and Pgp by immunohistochemistry using monoclonal antibody C219. The Cox regression model was used to adjust for several clinical and pathologic covariates.
**Results:** Of the 107 carcinomas examined, 44 (41.1%) were classified as DNA diploid and 63 (58.9%) as DNA aneuploid. DNA ploidy pattern was significantly related to tumor site ($P = 0.010$), tumor stage ($P = 0.016$), and vascular invasion ($P = 0.015$) but not to other clinicopathologic variables. Patients with DNA diploid tumors showed a better survival rate than did those with aneuploid tumors. After stage IV disease was excluded, patients with diploid tumors also presented a better disease-free and overall survival than did patients with aneuploid tumors. Mean SPF of the whole series was 13.5% (median = 11.3%, range = 1.4%–29.9%). Aneuploid tumors had a higher median SPF than did diploid tumors (17 *vs.* 6.2; $P = 0.0001$). SPF was only related significantly with tumor location ($P = 0.026$). In the multivariate analysis, SPF was a significant independent prognostic factor for overall survival ($P = 0.01$). When stage IV was excluded, SPF was also an independent prognostic variable for both disease-free ($P = 0.02$) and overall ($P = 0.01$) survival. Of 107 tumors, 61 (57%) were positive for Pgp expression, but no relation was found between this and other clinicopathologic parameters. Pgp expression had no influence on survival.
**Conclusions:** Our results suggest that flow cytometric DNA ploidy and SPF are significant and independent prognostic factors in patients with colorectal carcinoma, whereas Pgp expression is not.
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## INTRODUCTION

Colorectal carcinoma is the third leading cause of cancer death in most countries with a Western type of diet [1]. Surgical resection with curative intent is the primary treatment modality. However, nearly half of patients with colorectal cancer die of recurrent or metastatic disease. Significant variability in rates of recurrence and survival, particularly in early stages, highlights the need for other biological indicators of behavior to identify subsets of patients who may benefit from adjuvant therapies. At present, staging [2,3] is the only accepted and proven method of defining prognostic subsets of patients. Other clinicopathologic parameters, including age; bowel obstruction; perforation; tumor location, size, and histologic grade; vascular invasion; and degree of lymphocytic infiltrate [4–6], have been used to supplement staging. However, these parameters have far weaker prognostic correlations and often are not of independent predictive value.

The prognostic value of flow cytometric DNA ploidy in patients with colorectal carcinoma has not been defined clearly. Most investigators agree that the presence of aneuploid cell populations by flow cytometry is associated with reduced patient survival [7–14]. However, only in a limited number of studies has DNA ploidy status been demonstrated to be an independent prognostic variable by multivariate analysis including traditional prognostic parameters [6,15–18]. Moreover, other investigations have been unable to show any significant relation between clinical outcome and tumor DNA content [19–22].

A few studies have been undertaken in colorectal carcinoma to evaluate the prognostic value of S-phase fraction (SPF) for predicting survival, but results have been conflicting [6–8,20,23,24].

P-glycoprotein (Pgp), the *mdr* gene product, acts as an energy-dependent efflux pump that expels cytostatic drugs from the cell [25]. The principal focus of attention on Pgp has been its role in intrinsic and acquired anticancer drug resistance. However, certain properties of Pgp-rich epithelial cells in tissue culture support the hypothesis that Pgp expression may have an influence on cellular interactions and, therefore, on the biological behavior of cancers in vivo [26]. Thus, Pgp could be a marker of progression and may have a prognostic value independent of the drug-resistance mechanism.

The aims of the present study were, first, to examine the contribution of DNA ploidy, SPF, and Pgp expression as independent prognostic factors in colorectal carcinoma and, second, to assess their association with other clinicopathologic characteristics.

## MATERIALS AND METHODS

### Patients and Clinical Data

The study included 107 patients who underwent surgical resection at Vall d'Hebron Hospital of Barcelona (Spain) from January 1987 through December 1989. Fifty-nine (59/107 = 55.1%) were male and 48 (48/107 = 44.9%) were female, with a median age of 57.9 years (range = 34–78 years). Thirty-eight tumors were classified as stage II, 55 as stage III, and 14 as stage IV according to the International Union Against Cancer TNM classification [27]. Location of tumors was as follows: 27 in the right colon, 10 in the transverse colon, 12 in the left colon, 15 in the sigmoid colon, and 43 in the rectum. Fifty-seven patients with stage II and III disease received postoperative adjuvant chemotherapy, and radiotherapy when the rectum was involved. Patients with stage IV disease were treated with palliative chemotherapy. No patients received preoperative chemotherapy or radiotherapy. Information regarding clinical outcome was obtained from hospital chart review. Periodic assessment included a clinical examination, serum carcinoembryonic antigen test, endoscopy, abdominal computed tomography, and chest radiography.

During the observation period, 45 (42%) of 107 patients (13 with stage II and 32 with stage III disease) developed tumor recurrence (39 distant metastasis and 6 local recurrences); 36 of the 45 died (10 with stage II and 26 with stage III disease). All patients with stage IV disease died of the disease, and 5 patients died of causes unrelated to colorectal carcinoma without clinical evidence of recurrence. The mean duration of follow-up from the date of surgery to the date of death or the end of the study was 36.6 months (range = 3–72 months). None of the patients were lost to follow-up.

### Histopathologic Examination

Morphologic analysis on hematoxylin-eosin–stained sections was performed by a pathologist blinded to the clinical outcome. Blocks of paraffin-embedded tissue from all primary tumors, lymph nodes, and resected local recurrences or metastases were examined. Histologic type was determined according to the criteria of the World Health Organization [28]. Tumor grade was evaluated according to Jass et al. [4]. Tissue sections also were examined for the presence of vascular and perineural invasion.

### Flow Cytometric Analysis

Flow cytometry was performed on cell suspensions prepared from 50-μm sections from formalin-fixed paraffin-embedded tissue pertaining to the primary tumors,

lymph nodes, local recurrences, and metastases, as described previously [29]. At least 10,000 cells were scanned to construct each histogram. A histogram was considered interpretable if the coefficient of variation (CV) was <8%. The mean CV was 5.7% (range = 2.6%–8.0%). The DNA index (DI) was calculated by measuring the position of any aneuploid G1 peak relative to the normal G0/G1 peak, with a DI of 1.0 indicating the presence of only diploid cells. DNA aneuploidy was documented only if there was a clear evidence of a second G0/G1 peak on flow cytometric tracings. For DNA diploid tumors, the proportion of cells in SPF was calculated by the method of Baisch et al. [30]. For aneuploid tumors with a DI >1.2, a modification of this method was used to calculate the SPF for the aneuploid cells alone [31]. If the aneuploid peak accounted for ≤10% of the total number of cells, the SPF fraction was not calculated. Tetraploid tumors were included within the group of aneuploid tumors.

The flow cytometric study was performed on 120 tumors, but 2 cases resulted in uninterpretable histograms, and in 11 cases the SPF was not calculable. Thus, the study was confined to the 107 patients with known DNA ploidy and SPF.

### Pgp Immunohistochemistry

Immunohistochemical staining was performed on 5-μm formalin-fixed paraffin-embedded tissue sections containing both tumor and adjacent normal tissue. Expression of Pgp was assessed with the murine monoclonal antibody (MAb) C219 (Centocor Diagnostics, Malvern, PA). This MAb, subclass IgG2a, reacts against an epitope of Pgp present on the cytoplasmic side of the cell membrane and is 100% specific for Pgp [32].

Slides were dewaxed in xylene and rehydrated in alcohol. Endogenous peroxidase activity was blocked by incubation of slides in 20% phosphate-buffered methanol containing 0.2% hydrogen peroxide ($H_2O_2$) for 10 min. Deparaffinized sections were treated with 0.1% trypsin (Sigma, St. Louis, MO) in 0.15% calcium chloride (pH 7.8) at 37°C for 25 min. They were then washed several times in phosphate-buffered saline (PBS) and incubated with 10% normal horse serum–PBS for 20 min, followed by incubation with C219 murine MAb (it was used at 20 μg/ml in PBS containing 2% bovine serum albumin [BSA] for 1 h at 37°C). After a PBS rinse, the slides were incubated for 30 min with biotinylated horse anti–mouse IgG diluted 1:200 in BSA-PBS at 37°C. The slides were washed in PBS and placed for 30 min at room temperature in a solution of peroxidase conjugate streptavidin–biotin complex (DAKO LSAB kit, Copenhagen, Denmark) diluted 1:200 in 1% BSA-PBS. Diaminobenzidine (DAB) was used as chromogen (5 mg of DAB tetrahydrochloride in 100 ml of PBS containing 100 μl of 0.3% $H_2O_2$). Slides were incubated with DAB solution for 5–10 min. Between each step, the slides were washed three times for 10 min in PBS. After a counterstain with hematoxylin, the slides were dried and mounted.

Positive controls included paraffin-embedded sections from tumors found to express Pgp in a previous study [33]. For negative controls, sections were incubated in an isotype-matched negative control antibody at the same working dilution, provided in the C219 kit, instead of the primary antibody. The adjacent normal tissue was also used as control.

Three observers independently evaluated and interpreted the results of immunohistochemical staining without knowledge of the clinical data. A tumor was declared definitively negative when there was an absence of positive staining in any cell of any part of the tumor examined. Tumor samples were graded as 1 (<25% of positivity), 2 (25%–75% of positivity), or 3 (>75% of positivity) according to the degree of immunohistochemical staining of cells. The highest degree of positivity found was recorded. Staining intensities of the whole slide were graded as +, ++, or +++. Agreement between at least two observers was necessary for grading.

### Statistical Analysis

Calculations were done on a Vax computer using the MINITAB statistical analysis software. Comparison of ploidy, SPF, and expression of Pgp with the other clinicopathologic variables was performed by using the $\chi^2$ test for proportions. Disease-free and overall survival curves were compared by using the BMDP program 1L (Mantel–Cox). Multivariate analysis was performed with the BMDP program 2L (Cox proportional hazards regression model). A stepwise procedure with backward elimination was used to select variables that were independently associated with survival or relapse-free survival. At each step, only the variables having $P < 0.05$ were included in the model. Three Cox regression models were created to examine the prognostic significance of all the parameters in different stages of disease. The first model incorporated all patients of the study (n = 107) and analyzed the overall survival. The second model also examined survival but excluded stage IV disease (n = 93). The third model studied disease-free survival for stages II and III (n = 93).

## RESULTS

### Clinicopathologic Parameters and Survival

In univariate survival analysis of all 107 patients, female sex ($P = 0.02$), early stages ($P = 0.00009$), absence of vascular invasion ($P = 0.0002$), and adjuvant treatment ($P = 0.004$) were all significantly associated with better survival. The same results were obtained after excluding patients with stage IV disease (Table I). Similarly, the above-mentioned parameters, with the exception of sex, were correlated with higher disease-free sur-

**TABLE I. Univariate Analysis for Survival and Disease-Free Survival of Patients with Stages II and III According to Prognostic Factors (93 Patients)**

| | | Survival | | Disease-free survival | |
|---|---|---|---|---|---|
| Variable | N | No. of disease-related deaths (%) | $P^a$ | No. of patients with recurrence (%) | $P^a$ |
| Age | | | | | |
| <44 | 13 | 4 (30.8) | NS | 6 (46.2) | NS |
| 44–65 | 49 | 18 (36.7) | | 24 (49.0) | |
| >65 | 31 | 14 (45.2) | | 15 (48.4) | |
| Sex | | | | | |
| Male | 51 | 21 (41.2) | 0.04 | 29 (56.9) | NS |
| Female | 42 | 15 (35.7) | | 16 (38.1) | |
| Histologic type | | | | | |
| Adenocarcinoma | 84 | 33 (39.3) | NS | 44 (52.4) | NS |
| Mucinous | 9 | 3 (33.3) | | 1 (11.1) | |
| Histologic grade | | | | | |
| Well | 29 | 8 (27.6) | NS | 14 (48.3) | NS |
| Moderately | 47 | 18 (38.3) | | 24 (51.1) | |
| Poorly | 17 | 10 (58.8) | | 7 (41.2) | |
| Tumor location | | | | | |
| Cecum, right colon | 22 | 5 (22.7) | NS | 7 (31.8) | NS |
| Transverse colon | 8 | 2 (25.0) | | 4 (50.0) | |
| Left colon | 12 | 4 (33.3) | | 3 (25.0) | |
| Sigmoid | 12 | 5 (41.7) | | 7 (58.3) | |
| Rectum | 39 | 20 (51.3) | | 24 (61.5) | |
| Stage | | | | | |
| II | 38 | 10 (26.3) | 0.004 | 13 (34.2) | 0.0004 |
| III | 55 | 26 (47.3) | | 32 (58.2) | |
| Vascular invasion | | | | | |
| Yes | 34 | 20 (58.8) | 0.0005 | 24 (70.6) | 0.00009 |
| No | 59 | 16 (27.1) | | 21 (35.6) | |
| Perineural invasion | | | | | |
| Yes | 28 | 12 (42.9) | NS | 17 (60.7) | NS |
| No | 65 | 24 (36.9) | | 28 (43.1) | |
| Ploidy | | | | | |
| Diploid | 40 | 8 (20.0) | 0.0025 | 11 (27.5) | 0.0002 |
| Aneuploid | 53 | 28 (52.8) | | 34 (64.2) | |
| S-phase fraction | | | | | |
| ≤11.3% | 48 | 13 (27.1) | 0.016 | 14 (29.2) | 0.00008 |
| >11.3% | 45 | 23 (51.1) | | 31 (68.9) | |
| P-glycoprotein primary tumor | | | | | |
| Positive | 48 | 23 (47.9) | 0.06 | 28 (58.3) | 0.07 |
| Negative | 45 | 13 (28.9) | | 17 (37.8) | |

[a]NS = Not significant, i.e., $P > 0.05$.

vival (Table I). Conversely, age, tumor location, histologic tumor grade, histologic type, and perineural invasion did not influence survival. In the first Cox regression model, the only parameters that remained as independent prognostic factors for overall survival were stage ($P = 0.04$) and vascular invasion ($P = 0.04$). In models 2 and 3, sex and adjuvant treatment, respectively, were added to these parameters as independent variables (Table II).

## DNA Ploidy and Clinicopathologic Features

Of the 107 carcinomas examined, 44 (41.1%) were DNA diploid and 63 (58.9%) were DNA aneuploid. The mean CV of the G0/G1 peak was 5.7% (median = 5.4%; range = 2.6%–8%). The DI ranged from 1.2 to 2 in 93.4% of aneuploid tumors, and only 4 tumors showed a DI >2. No relation was found between DNA ploidy and patient age, sex, histologic type, tumor grade, or perineural invasion. However, DNA ploidy was related to tumor location ($P = 0.010$), tumor stage ($P = 0.016$), and vascular invasion ($P = 0.015$; Table III). Tumors of the sigmoid colon and rectum were more frequently DNA aneuploid than those located proximally to the splenic flexure. Furthermore, an aneuploid cell population was detected more often in stage III and IV disease and in tumors with vascular invasion.

## DNA Ploidy and Survival

In univariate survival analysis, patients with DNA diploid tumors had a better overall survival ($P = 0.00008$)

**TABLE II. Multivariate Analysis of Prognostic Factors for Overall Survival in Stages II, III, and IV (107 Patients)**

| Variable | Hazard rate (95% confidence interval) | *P* |
|---|---|---|
| Model 1: stages II, III, and IV (n = 107) | | |
| Stage | | |
| II | 1.00 | |
| III | 2.18 (1.00–4.74) | 0.04 |
| IV | 3.19 (1.16–8.75) | |
| Ploidy | | |
| Diploid | 1.00 | 0.003 |
| Aneuploid | 3.31 (1.36–8.04) | |
| S-phase fraction | | |
| ≤11.3% | 1.00 | |
| >11.3% | 1.61 (1.00–1.24) | 0.01 |
| Vascular invasion | | |
| Yes | 1.94 (1.00–3.76) | 0.04 |
| No | 1.00 | |
| Model 2: stages II and III (n = 93) | | |
| Stage | | |
| II | 1.00 | 0.04 |
| III | 1.92 (0.87–4.25) | |
| Ploidy | | |
| Diploid | 1.00 | 0.007 |
| Aneuploid | 3.18 (1.28–7.90) | |
| S-phase fraction | | |
| ≤11.3% | 1.00 | |
| >11.3% | 1.82 (1.14–1.83) | 0.01 |
| Vascular invasion | | |
| Yes | 2.88 (1.33–6.22) | 0.0065 |
| No | 1.00 | |
| Sex | | |
| Male | 1.37 (0.56–2.88) | 0.02 |
| Female | 1.00 | |
| Model 3: stages II and III (n = 93) | | |
| Stage | | |
| II | 1.00 | 0.0087 |
| III | 2.42 (1.21–1.84) | |
| Ploidy | | |
| Diploid | 1.00 | 0.002 |
| Aneuploid | 3.20 (1.46–7.03) | |
| S-phase fraction | | |
| ≤11.3% | 1.00 | |
| >11.3% | 1.87 (1.00–2.55) | 0.02 |
| Vascular invasion | | |
| Yes | 2.62 (1.35–5.06) | 0.0039 |
| No | 1.00 | |
| Adjuvant treatment | | |
| Yes | 1.00 | |
| No | 2.38 (1.18–4.78) | 0.0074 |

than did patients with aneuploid tumors, and this did not change, even for disease-free survival, when stage IV disease was excluded (Table I). In the 3 models of the multivariate analysis, DNA ploidy was a significant independent prognostic factor for both tumor recurrence and survival (Table II). It is noteworthy that, in patients with DNA aneuploid tumors, the risk of recurrence and cancer-related death was more than three times higher than that in patients with DNA diploid tumors, even after adjustment for TNM stage and other clinicopathologic variables.

## SPF and Clinicopathologic Features

The mean SPF of the whole series was 13.5% (median = 11.3%; range = 1.4%–29.9%). Patients were divided into 2 groups by using the median value of SPF as a cutoff point. Aneuploid tumors had a higher median SPF (17.1; range = 3.0–29.9) than did diploid tumors (6.2; range = 1.4–16.4), a statistically significant difference ($P = 0.00006$). There was no association of SPF with age, sex, tumor grade, histologic type, perineural invasion, tumor stage, or vascular invasion. However, SPF was significantly related to tumor location ($P = 0.026$; Table III). Tumors in the sigmoid colon and rectum had a higher SPF than did those located proximally to the splenic flexure.

## SPF and Survival

In univariate survival analysis, patients with an SPF ≤11.3 had a better overall survival ($P = 0.0028$) than did patients with greater SPF values. After excluding stage IV disease, patients with SPF ≤11.3 also showed a better disease-free and overall survival (Table I). In the 3 models of the multivariate analysis, SPF was a significant independent prognostic factor for both tumor recurrence and survival (Table II). The risk of recurrence and cancer-related death was higher in patients with SPF >11.3 than in patients with SPF ≤11.3, even after adjustment for TNM stage, other clinicopathologic variables, and DNA ploidy.

## Pgp Expression and Clinicopathologic Features

Sixty-one tumors (61/107 = 57%) were positive for Pgp expression, and 46 (46/107 = 43%) were Pgp negative. Twenty-two tumors showed positive expression grade 1, 23 grade 2, and 16 grade 3, and the staining intensity was + in 21, ++ in 18, and +++ in 22 preparations. Pgp expression in lymph nodes was analyzed in 69 cases and was positive in 41 (59.4%). In 57 cases, the expression of Pgp in lymph nodes was similar to that of the primary tumor ($P = 0.00007$), whereas in 12 cases it was different (6 cases positive in tumor and negative in lymph nodes and vice versa in the remaining cases). Pgp expression was also analyzed in 26 liver metastasis and local recurrences and was found to be positive in 14 cases (53.8%). In 20 cases expression was the same as that of the primary tumor, but in 6 it was different (4 cases positive for the tumor and negative for the metastasis and vice versa in the rest). No relation was found between Pgp expression and patient age, sex, tumor location, histologic type, grade of differentiation, perineural invasion, ploidy, or SPF. Of marginal significance were tumor stage ($P = 0.054$) and vascular invasion ($P = 0.056$).

**TABLE III. Correlation between Histopathologic Characteristics and Flow Cytometric Data**

| | No. | Ploidy, no. (%) Diploid | Aneuploid | $P^a$ | S-phase, no. (%) ≤11.3% | >11.3% | $P^a$ |
|---|---|---|---|---|---|---|---|
| Age, years | | | | | | | |
| <44 | 15 | 8 (53.3) | 7 (46.7) | NS | 8 (53.3) | 7 (46.7) | NS |
| 44–65 | 58 | 22 (37.9) | 36 (62.1) | | 28 (48.3) | 30 (51.7) | |
| >65 | 34 | 13 (38.2) | 21 (61.8) | | 16 (47.1) | 18 (52.9) | |
| Sex | | | | | | | |
| Male | 59 | 23 (39.0) | 36 (61.0) | NS | 30 (50.8) | 29 (49.2) | NS |
| Female | 48 | 20 (41.7) | 28 (58.3) | | 22 (45.8) | 26 (54.2) | |
| Tumor site | | | | | | | |
| Cecum, right colon | 27 | 17 (63.0) | 10 (37.0) | 0.010 | 18 (66.7) | 9 (33.3) | 0.026 |
| Transverse colon | 10 | 3 (30.0) | 7 (70.0) | | 4 (40.0) | 6 (60.0) | |
| Left colon | 13 | 8 (61.5) | 5 (38.5) | | 10 (76.9) | 3 (23.1) | |
| Sigmoid | 16 | 4 (25.0) | 12 (75.0) | | 6 (37.5) | 10 (62.5) | |
| Rectum | 41 | 11 (26.8) | 30 (73.2) | | 15 (36.6) | 26 (63.4) | |
| Histologic type | | | | | | | |
| Adenocarcinoma | 97 | 41 (42.3) | 56 (57.7) | NS | 46 (47.4) | 51 (52.6) | NS |
| Mucinous | 10 | 3 (30.0) | 7 (70.0) | | 6 (60.0) | 4 (40.0) | |
| Grade | | | | | | | |
| Well | 33 | 9 (27.3) | 24 (72.7) | NS | 13 (39.4) | 20 (60.6) | NS |
| Moderately | 53 | 24 (45.3) | 29 (54.7) | | 27 (50.9) | 26 (49.1) | |
| Poorly | 21 | 10 (47.6) | 11 (52.4) | | 12 (57.1) | 9 (42.9) | |
| Stage | | | | | | | |
| II | 38 | 22 (57.9) | 16 (42.1) | 0.016 | 21 (55.3) | 17 (44.7) | NS |
| III | 55 | 18 (32.7) | 37 (67.3) | | 26 (47.3) | 29 (52.7) | |
| IV | 14 | 3 (21.4) | 11 (78.6) | | 4 (28.6) | 10 (71.4) | |
| Vascular invasion | | | | | | | |
| Present | 45 | 12 (26.7) | 33 (73.3) | 0.015 | 18 (40.0) | 27 (60.0) | NS |
| Absent | 62 | 31 (50.0) | 31 (50.0) | | 33 (53.2) | 29 (46.8) | |
| Perineural invasion | | | | | | | |
| Present | 35 | 14 (40.0) | 21 (60.0) | NS | 16 (45.7) | 19 (54.3) | NS |
| Absent | 72 | 30 (41.7) | 42 (58.3) | | 37 (51.4) | 35 (48.6) | |

[a]NS = not significant, i.e., $P > 0.05$.

### Pgp Expression and Survival

In univariate survival analysis, patients with positive Pgp expression had a trend toward a poorer prognosis, even though the results were not statistically significant ($P = 0.06$). The same occurred when patients with stage IV disease were excluded, even for disease-free survival (Table I). In the 3 models of the multivariate analysis, there was no association of Pgp expression with either disease-free or overall survival (Table II).

## DISCUSSION

In our study, we screened for DNA ploidy, SPF, and Pgp expression in paraffin-embedded sections of 107 colorectal cancers to clarify the relation of these parameters with the clinical outcome. We found that 58.9% (63/107) of tumors had an aneuploid DNA content, in agreement with findings in previous studies [34–36]. In both our study and some previous studies [36], but in contrast to the finding of Dean and Vernava [35], a significant ($P = 0.016$) association between DNA ploidy and pathologic stage was found, with an increased occurrence of aneuploid tumors in patients with advanced disease. Therefore, our results reinforce the hypothesis that DNA ploidy may be associated with progression of colorectal carcinoma. DNA ploidy was also associated with vascular invasion ($P = 0.015$), in disagreement with previous reports [14,36]. As in other studies [12,14,37,38], we found a close relation between nuclear DNA content and anatomic site ($P = 0.010$). Tumors of the right and transverse colon were more frequently DNA diploid than those located distally to the splenic flexure. In fact, recent investigations have demonstrated the existence of biological differences (e.g., allelic deletions, p53 gene mutations, microsatellite instability) between tumors of the proximal and distal colon [39–42]. Finally, similar to previous reports [23,36], in our series DNA ploidy was not related to other clinicopathologic parameters.

As far as survival is concerned, the majority but not all [19,21,22] of previous flow cytometric studies have concluded that patients with DNA diploid tumors have a better survival than do those patients with aneuploid tumors [6–9,14–16]. The results of the current study support this view, and of note, DNA ploidy was the most significant independent predictor of disease-free ($P = 0.002$) and overall ($P = 0.003$) survival in the multivari-

ate analysis. Therefore, DNA ploidy status allows the discrimination of patients with different risks of recurrence and cancer-related death.

In spite of the potential clinical use of cell proliferation as an indicator of tumor growth, flow cytometric studies on SPF in colorectal carcinoma are far less numerous than those concerning DNA ploidy status [6–8,20,23,24,36]. In our study, we found the mean SPF of the whole series to be 13.5%, which is similar to findings of others [6]. We also demonstrated a statistically significant correlation between SPF and tumor location ($P = 0.026$).

Several studies have shown that aneuploid colorectal cancers have a considerably higher SPF than do diploid tumors, and our data confirm this observation. The close relation between aneuploidy and high SPF may raise the question of whether the prognostic significance of SPF could be due to its correlation with ploidy, thereby eliminating its value as an independent parameter of disease outcome [12,17,22,43]. However, as in other investigations [6,8,24], our data underscore the fact that high proliferative activity is a powerful adverse prognostic indicator for disease-free and overall survival. In multivariate analysis, we observed that SPF was a stronger prognostic factor than stage with regard to survival but not to recurrence.

In the present study, expression of Pgp was detected in 57% (61/107) of the primary colorectal carcinomas, a percentage similar to those previously reported [26,44,45]. We found a weak relation between Pgp and stage, with an increased incidence of Pgp-positive expression in advanced disease stages, as did D'Incalci et al. [46]. Therefore, Pgp could be a marker of progression, and this is supported by our observation that the staining of Pgp in lymph nodes tended to correlate with the staining of the primary tumor, as in the study of Weinstein et al. [26]. Mizoguchi et al. [47] found that *MDR1* mRNA was expressed more in well differentiated than in moderately differentiated colorectal carcinomas, but we did not find a statistically significant association between Pgp expression and grade or the other clinicopathologic parameters.

Weinstein et al. [26] found that Pgp expression was localized predominantly to invasively growing tumor cells, suggesting that Pgp expression was associated with local tumor aggressiveness and, hence, could be related to the clinical outcome. Despite these observations, we and others [44,48] found that neither *MDR1* mRNA nor Pgp expression of the tumors was of prognostic value in patients with colorectal carcinomas.

One limitation of our study should be noted, namely the use of paraffin-embedded material. It was recently stated that fresh or frozen material is preferable to paraffin-embedded tissue for DNA ploidy and SPF analysis by flow cytometry, which is also applicable to Pgp detection. However, the ability to assess these parameters in formalin-fixed, paraffin-embedded samples has the advantage of allowing studies to be performed on large retrospective series of cases.

## CONCLUSIONS

The results of the current study confirm that flow cytometric DNA ploidy and SPF are significant and independent prognostic factors in patients with colorectal carcinoma. This should permit DNA ploidy and SPF to be used for selecting for adjuvant therapy a subset of stage II patients with a poor prognosis (patients with high SPF and aneuploid tumors). Thus, we can justify the routine practice of flow cytometric analysis of DNA ploidy and SPF in human colorectal carcinomas for the purpose of prognostication. Furthermore, this study also bolsters the clinical importance of the assessment of pathologic tumor stage and vascular invasion as factors with prognostic significance in colorectal carcinoma. Pgp expression seems to be of no value for this purpose.

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